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Adhesive and growth properties of lectin from the ascidian *Didemnum ternatanum* on cultivated marine invertebrate cells

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Abstract

The effects of *N*-Acetyl-D-glucosamine-specific lectin (M_r 27 kDa) isolated from the ascidian *Didemnum ternatanum* on cultivated cells of molluscs and echinoderms were studied. This lectin was found to stimulate the growth or the differentiation of cultivated marine invertebrate cells depending on the stage of embryonic development at which primary cell cultures were obtained. In addition, it has been shown to increase the attachment of cells in primary cultures of these animals. The degree of attachment is considerably increased when collagen or polylysine substrates are used. Using scanning electron microscopy we have demonstrated the stage-specific effect of this lectin on embryonic sea urchin and molluscan cells. Intensive cell spreading and an alteration of cell shape were observed only at the gastrula stage, when the switching from maternal information to embryonic genes occurred. The ascidian lectin seems to have some characteristics of both an adhesive factor and a growth factor.

Keywords: Ascidian lectin; Adhesion; Growth factor; (Mussel); (Sea urchin)

1. Introduction

There are some hundreds of cell lines from tissues of invertebrates, most of them are the lines of insect cells. All efforts to obtain continuous cell line of molluscs, echinoderms or crustaceous have so far failed, although cultures of viable molluscan cells have been maintained for different time intervals [1–6]. Only one line of embryos of the freshwater mollusc *Biomphalaria glabrata* has been described [7]. Unfortunately, all cells in primary cultures of marine invertebrates have a low proliferative level; cell degeneration predominates over proliferation even in the embryonic or larval cultures [5,8–11]. The lack of stimulators of cell proliferation (growth factors) and specific adhesive factors can be a cause of failure in long-term cultivation of these cells. Embryonic cells of molluscs and echinoderms, cultivated in vitro, are model cell systems with a high level of all physiological and synthetic processes and are very sensitive to the influence of different medium components. In this work we investigated the effects of GlcNAc-specific lectin from the ascidian *Didemnum*

Abbreviations: DTL, *Didemnum ternatanum* lectin; GlcNAc, *N*-acetyl-D-glucosamine; BSA, bovine serum albumin; Con A, concanavalin A; Met-Man, methyl-D-mannoside; FITC, fluorescein isothiocyanate; ILN, index of labelled nuclei

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ternatanum on cultivated cells of molluscs and echinoderms. Lectins are multivalent carbohydrate-binding proteins, which can be used as the tools for modification of cell function through specifically interaction with cell membrane glycoconjugates [12]. They have been implicated as controlling molecules in cell aggregation, differentiation, invertebrate immune response, growth and division [12,13].

In an earlier paper [14] we reported on the effects of DTL on HeLa-M tumour cells. This Ca^{+2}-independent lectin (M_r 27 kDa) resulted in a marked increase of adhesiveness and an intensive cell spreading of the transformed cells. The question about adhesive capability of DTL for marine invertebrate cells has been discussed in our previous work [15].

2. Materials and methods

2.1. Materials

Sea urchins (*Strongylocentrotus nudus* and *S. intermedius*) and the mussel (*Mytilus trossulis)* were collected in Vostok Bay, the Sea of Japan. The animals were washed 2–3 times with ultraviolet (UV)-irradiated seawater and maintained in tanks with aerated UV-irradiated seawater at 14–15°C for 2 days. The embryonic material for primary cell cultures was obtained by artificial fertilisation of these animals. Spawning was induced by an electric shock (8–12 V) or thermal shock. The embryos were placed in closed tanks with UV-sterilised seawater (15°C).

DTL was isolated from the colonial ascidian *Didemnum ternatanum* and purified by affinity chromatography on cross-linked ovalbumin followed by gel-filtration on Sephadex G-100 [16]. Sterile solutions of DTL (500 μg/ml) were added into the wells to the final concentration of 2.5 μg/ml (this concentration was chosen after preliminary experiments in which the effect of DTL on cell viability was tested in the concentration range of 0.1–10.0 μg/ml).

2.2. Cell cultures

Mussel larvae at the sterroblastrula and trochophore stages (16 and 28 h after fertilisation, respectively), and sea urchin larvae at the blastula and gastrula stages (10 and 28 h after fertilisation, respectively), were collected onto a finely-meshed gauze, washed in Ca^{2+}- and Mg^{2+}-free artificial sea water (CMFSS) containing EDTA (15 mM) and antibiotics (100 IU:100 μg/ml penicillin/streptomycin), then washed several times in sterile seawater with the same antibiotics.

The embryos were dissociated in 0.125% collagenase (isolated from the liver of the crab *Paralithodes camtschatica* in the Pacific Institute of Bioorganic Chemistry, Far East Branch, Russian Academy of Sciences) for 20–40 min at 15°C. The resulting cell suspension containing all cell types was washed twice by centrifugation (1500 rpm, 10 min) in CMFSS containing EDTA (15 mM) and gentamycin (40 μg/ml). The cell precipitate was resuspended in a modified Leibovitz medium [10] supplemented with 2% foetal calf serum, vitamin E (1.75 mg/l), insulin (5 mg/l) and gentamycin (40 mg/l). Cells were cultivated in 24-well culture plates (Limbro) at 15°C. Seeding concentration was 1.8–2.4 10^6 cells/ml. Cell viability was estimated by simultaneous staining with fluorescein diacetate and ethidium bromide [17]. At the time of seeding, cell viability was 90–95% for echinoderm cultures and 80–90% for molluscan cultures, and it slightly declined (to 75–85%) on day 10.

2.3. Scanning microscopy

Cells grown on microfilters (Millipore, 0.22 μm) were incubated for 72 h in a medium supplemented with or without DTL in a final concentration of 2.5 μg/ml. Cells were then fixed in 2.5% glutaraldehyde (Serva) in seawater for 30 min at 15°C. Preparations were subsequently dehydrated in an ethanol series, critical point-dried, and coated with gold. Samples were analysed using an ISM-25-CF (Jeol) scanning microscope and photographed at a tilt angle of 30°.

2.4. Adhesion assay

The number of adherent cells was determined as the difference between cell number that was originally plated into the well and the number of non-adherent cells in each well after 3 days of cultivation. The cell density of the suspension of non-adherent cells was counted by visually in a counting chamber. Uncoated (plastic) wells were used as control. The

percentage of attached cells was estimated as the number of attached cells in wells relative to the cell number that was originally plated into the well (100%). The following substrates were tested in this study: poly-L-lysine (M_r 190 kDa, Serva); fibronectin (Serva), collagen (type 1, Sigma) and DTL. Proteins were diluted (1 mg/ml) in a phosphate buffer (pH 7.4), except DTL, which was dissolved in water at 0.5 mg/ml. The protein solution (0.3 ml) was plated onto each well and allowed to store for 3–4 h. After washing (twice in 1 ml of sterile seawater), plates were stored for up 24 h at 4°C. In some tests, DTL or Con A were directly added to the medium for a final concentration of 2.5 μg/ml and 1.5 μg/ml, respectively. Specific sugars, GlcNAc (80 mM) for DTL and Met-Man (80 mM) for Con A, were used as inhibitors. Experiments were run in triplicate, and mean values with standard errors were provided.

2.5. Scintillation procedure for measuring [^{3}H]thymidine and [^{3}H]uridine incorporation

Cells were plated at 5×10^5 cells per well and incubated at 15°C for 3 days. Then labelled precursors ([^{3}H]thymidine, 5 μCi/ml, specific activity 83 Ci/mmol or [^{3}H]uridine, 10 μCi/ml, specific activity 0.7 Ci/mmol) were added to the cell suspension. Cells were incubated for 10–12 h at 15°C, after that the medium was changed to fresh with unlabelled nucleosides for 15 min. The contents of the wells were quantitatively deposited on the membrane filters (Millipore, 0.22 μm). Additionally, the wells were washed twice with CMFSS containing EDTA (15 mM). To remove material that non-specifically binds to the cells, the filters were washed in 10% ice-cold trichloroacetic acid (10 ml) followed by ethanol and put into vials with 5 ml of a toluene scintillator. Radioactivity was measured on a LC-30 counter (Intertechnique, France). In each case, experiments were run in triplicate.

2.6. Autoradiography

Embryonic cells were cultivated for 3 days on coverslips and then [^{3}H]thymidine was added to the medium (final concentration 5 μCi/ml). A day later, the coverslip cultures were washed twice in seawater and incubated with unlabelled thymidine for 1 h at 15°C. Cells attached to the coverslips were fixed for 30 min in vapours of 40% formaldehyde and washed twice in water. The coverslips were consecutively dehydrated in an ethanol series, covered with photoemulsion and exposed for 2 months at 4°C. After the autographs were developed, the coverslips were stained with Carazzi haematoxylin and eosin. DNA-synthetic activity was determined by counting the number of thymidine-labelled nuclei per 500–1000 cells and designated as the index of labelled nuclei (ILN) expressed in percentages. The intensity of label was evaluated by the quantity of silver grains above the nucleus. All nuclei were divided into three groups: unlabelled (less than 5 grains, background); weakly labelled (5–10 grains per nucleus); and intensely labelled (over 10 grains). Assays were performed in triplicate or quadruplicate.

2.7. Fluorescent microscopy

Lectins, DTL and Con A, and bovine serum albumin (BSA) as control, were labelled with FITC at 4°C using a procedure modified from that described by Rao [38]. Embryonic cells were incubated at 4°C with labelled agents for 30 min, then they were washed three times in seawater and incubated for 15 min at room temperature. Cells were fixed in 3% formaldehyde for 10 min at room temperature and washed twice in seawater before microscopy. Fluorescent microscopy was performed by using a Polyvar microscope (Reichert–Jung, Germany) equipped with a Ploem vertical illuminator containing the standard filter set.

2.8. Determination of binding constants

To determine constants of DTL binding to sea urchin and molluscan cells at different embryonic stages, FITC-DTL was used. The aliquot (500 μl) of various concentration of FITC-DTL solution at seawater buffered with 0.05 M sodium phosphate (pH 7.4) contained 1% BSA was added to 500 μl of cell suspension (2×10^5 cells/ml), and mixture was incubated at 37°C for 2 h. The binding lectin was determined as fluorescence difference of added lectin, and remained in solution after centrifugation at $500\times g$ for 10 min at 25°C. The fluorescence at 517 nm with excitation at 493 nm was recorded on a

fluorescent spectrophotometer (M-850, Hitachi, Japan). Specific binding was confirmed in each experiment by adding 10 mM GlcNAc. The binding constants were calculated by using method of Matsumoto with colleagues [18] as follows: $C/\Delta F = 1/K_a * 1/\Delta F_{max}, -C * 1/\Delta F_{max}$, where ΔF is fluorescence of binding DTL, C the concentration of FITC-labelled DTL added, and ΔF_{max} was determined from double reciprocal curve $1/\Delta F = f(1/C)$ by extrapolation to $1/C = 0$.

3. Results

3.1. Scanning microscopy data

DTL can significantly alter the adhesion and morphology of embryonic sea urchin and mussel cells, depending on the stage of embryonic development at which primary cultures were initiated (Figs. 1 and 2). Sea urchin blastula cells cultivated in a medium supplemented with DTL did not change their morphology (Fig. 1a,b), while gastrula cells (for molluscs, cells of the trochophore stage), becoming well spread, formed a cell layer (Fig. 1d, Fig. 2d), as opposed to the control cells that remained desegregated and preserved a spherical shape (Fig. 1c, Fig. 2a). Cell shape in primary culture from the gastrula or trochophore stages began to change already after 1 h of incubation with DTL (Fig. 2b), and after 5 h of incubation there were extensive regions of spread cells (Fig. 2c).

3.2. Cell attachment

In addition to the results from morphological ob-

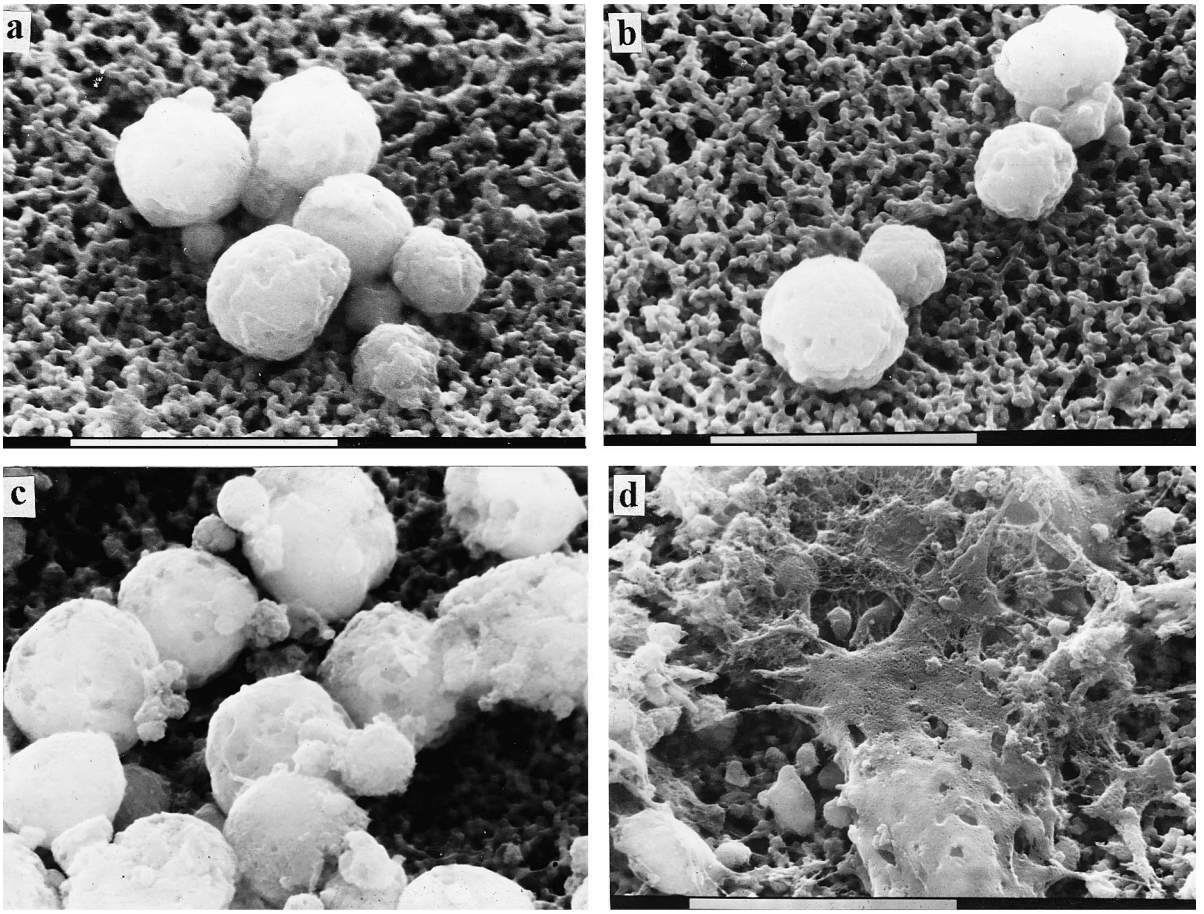

Fig. 1. Effect of DTL on the embryonic cells of *Strongylocentrotus nudus* (scanning electron microscopy). (a,b) Blastula cells. (c,d) Gastrula cells: c, untreated (control); d, cells cultured with DTL (2.5 μg/ml). All cultures were grown at 15°C on filters (Millipore, 0.22 μm) for 3 days prior to fixation. Magnification is the same for all panels. Bar = 10 μm.

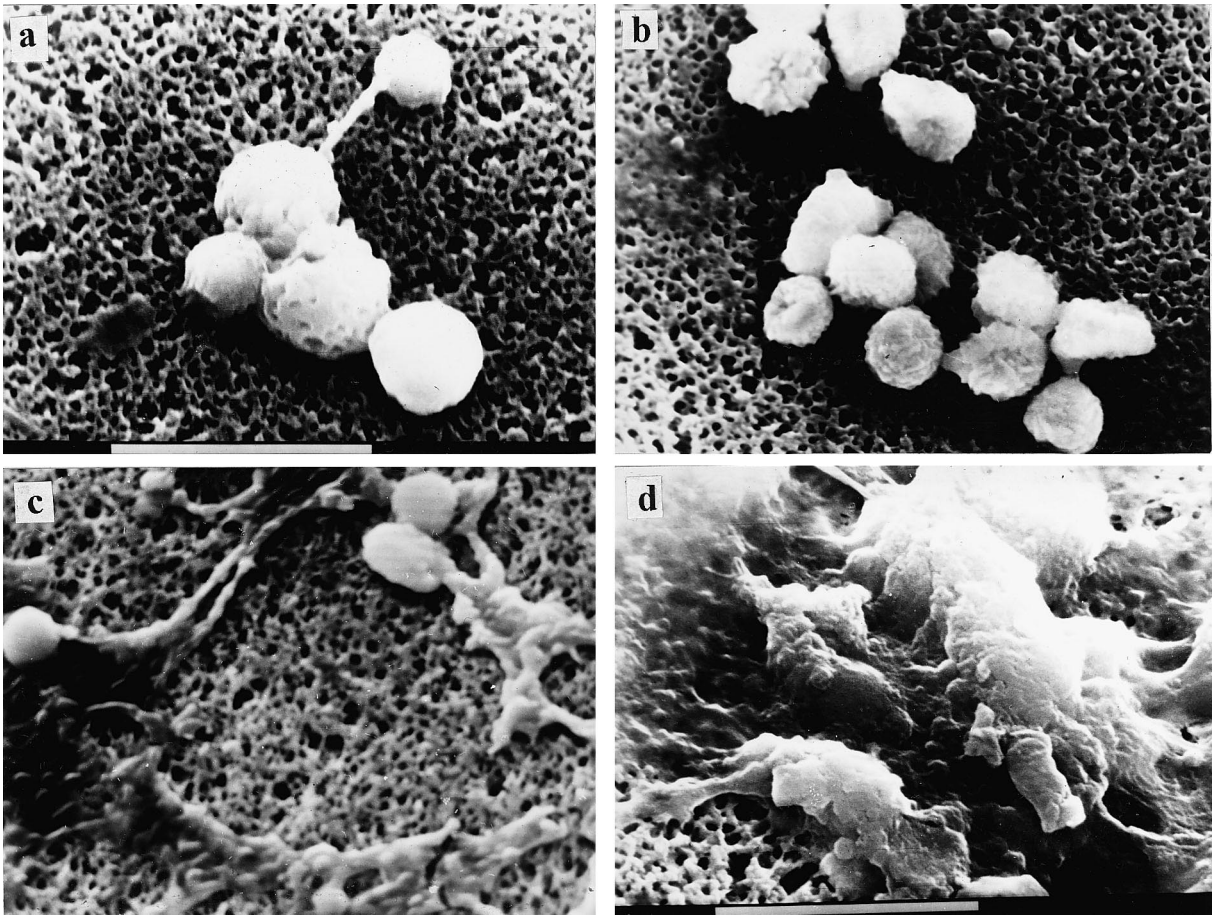

Fig. 2. Time-effect of DTL on the trochophore cells of *Mytilus trossulus* (scanning electron microscopy). (a) Untreated (control) cells. Cells after incubation with DTL (2.5 μg/ml): (b) for 1 h; (c) for 5 h; (d) for 3 days. All cultures were grown at 15°C on filters (Millipore, 0.22 μm) prior to fixation. Magnification is the same for all panels. Bar = 10 μm.

servations, there are data on the cell attachment of embryonic sea urchin and molluscan cells on the different substrates (Fig. 3). DTL increases the attachment of both echinoderm and molluscan cells obtained at the gastrula or trochophore stages. Cell attachment is considerably increased when collagen or poly-L-lysine substrates are used. DTL-induced cell adhesion is not inhibited completely by the addition of specific sugar, GlcNAc, whereas cell adhesion by the other lectin, Con A, is inhibited essentially by the addition of Met-Man.

3.3. [^{3}H]Thymidine incorporation

The level of [^{3}H]thymidine incorporation in cells cultivated in poly-L-lysine-coated wells is correlated with the data on plating efficiently and exceeds more than 2-fold the control values (Table 1). However, there is no correlation when cells are incubated with DTL: the treatment of cells cultured for 3 days with DTL produce significantly increased (3–5-fold) [^{3}H]thymidine incorporation into blastula and gastrula cells.

Analysis of the autoradiographic material revealed a fairly high ILN in the embryonic cells of sea urchin (Table 1). Significant differences were found between the cultures initiated at the different stages of embryogenesis. The ILN was 9% for cell cultures obtained at the blastula stage and 41% for cells obtained at the gastrula stage. In both the embryonic culture systems, DTL increased the ILN, and especially strongly in blastula cells, i.e., 3-fold.

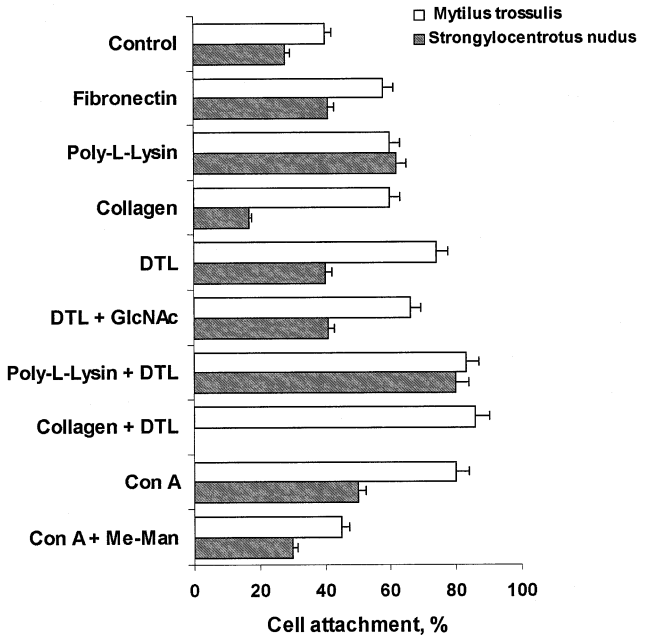

Fig. 3. Cell attachment (%) of sea urchin and mussel cells at the stage of the gastrula (trochophore) on different substrates. Cell attachment (in %) was estimated as the number of adherent cells in wells to the cell number that was originally plated into the well (100%). The number of adherent cells was determined as the difference between the initial cell number and the number of non-adherent cells in each well after 3 days of cultivation. The cell density of the suspension of non-adherent cells was counted visually in a counting chamber. Uncoated (plastic) wells were used as control. Mean values and standard errors are provided.

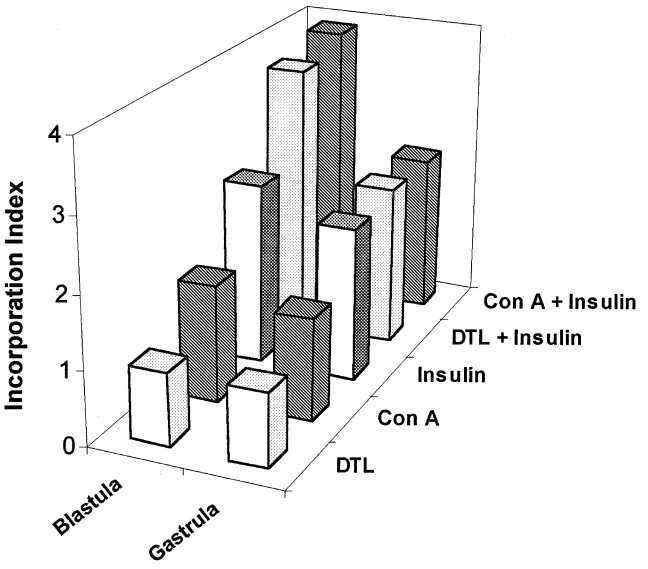

Fig. 4. Influence of the lectins and insulin on the RNA synthesis in primary cell cultures from sea urchin larvae. The level of [^{3}H]uridine incorporation in control wells (plastic) was taken to be 1. Concentrations of the lectins used: DTL, 2.5 μg/ml; Con A, 1.5 μg/ml. Insulin concentration 100 μg/ml.

3.4. [^{3}H]Uridine incorporation

DTL (as did Con A) exerted no effect on the RNA synthesis in cells of embryonic cultures. However, in the presence of one of the growth factors, insulin, which itself results in a 2-fold increase of the level of [^{3}H]uridine incorporation, lectins increased additionally by two times this level, but only for blastula cells (Fig. 4).

3.5. DTL binding constants with cell surface carbohydrates

DTL binding activity with terminal GlcNAc residues on the surface of molluscan and sea urchin cells was different and not dependent on the stage of embryonic development (Table 2). The high affinity of

Table 1
Intensity of DNA synthesis in embryonic cells of the sea urchin *Strongylocentrotus nudus*, cultivated on different substrates[a]

Substrate	Level of [^{3}H]thymidine incorporation		ILN, %	
	blastula	gastrula	blastula	gastrula
Control	1.0*	1.0*	9 ± 1.2	41 ± 1.2
Poly-L-lysine	2.3 ± 0.2	2.40 ± 0.20	34 ± 0.1	45 ± 5.0
Fibronectin	–[b]	1.92 ± 0.18	28 ± 1.7	24 ± 1.4
DTL	5.5 ± 0.4	3.44 ± 0.44	27 ± 7.5	50 ± 6.3
DTL in medium (2.5 μg/ml)	6.0 ± 0.08	3.00 ± 0.50	–	–

The cells were cultured for 3 days at 15°C.
[a]The level of [^{3}H]thymidine incorporation in control wells (plastic) was taken to be 1.
[b]Not determined.

Table 2
The constants (mM^{-1}) of DTL binding to embryonic cells at various developmental stages

	Eggs	Blastula	Gastrula (trochophore)
Mytilus trossulis	67.4 ± 2.6	209.4 ± 4.7	198.4 ± 3.9
Strongylocentrotus nudus	1.3 ± 0.1	1.0 ± 0.2	1.3 ± 0.2

DTL binding with mussel cells ($K_a \sim 200\ mM^{-1}$) was observed at all embryonic stages. Conversely, this lectin showed a low affinity for sea urchin cells (K_a 1–1.3 mM^{-1}).

3.6. Cell localisation of DTL

As shown by the fluorescence microscopic assay, the specific localisation of DTL at the blastula stage appeared to be largely associated with the cell membranes: 'patching'- and 'capping'-like phenomena were observed at the cell surface of mollusc and echinoderm cells (Fig. 5a,c). At the gastrula stage, the membrane localisation of DTL without 'capping-like' effects was found for sea urchin cells (Fig. 5b), while at the trochophore stage the penetration of DTL into mussel cells was shown (Fig. 5d). The BSA controls showed only minimal background fluorescence with no specific labelling.

4. Discussion

DTL was found to increase the attachment of molluscan, and echinoderm cells and to result in intensive cell spreading and an alteration of cell shape only at the gastrula stage, when the genes responsible for differentiation began to work. In addition, it was shown to stimulate the growth or the differentiation of cultivated marine invertebrate cells depending on the stage of embryonic development at which primary cell cultures were obtained. The mechanisms governing these adhesive changes and the specific functions they serve in development are not currently understood.

Cell adhesion by DTL is not inhibited completely by the addition of specific sugar GlcNAc. Conversely, cell adhesion by the other lectin, Con A, is decreased essentially by the addition of specific sugar. These data agree with the results of Mafranga et al. [19] and Latham et al. [20], who reported that Con A-induced adhesion in embryonic sea urchin cells was determined by a carbohydrate-recognition mechanism. It is possible that the adhesive activity of DTL is not due to only carbohydrate-binding site, but rather to other features of this lectin. Recently, we have demonstrated the ability of DTL to interact with collagen (type I); this interaction is inhibited by Arg–Gly–Asp (RGD)-peptide and peptides obtained from collagen (data not shown). It is possible, that

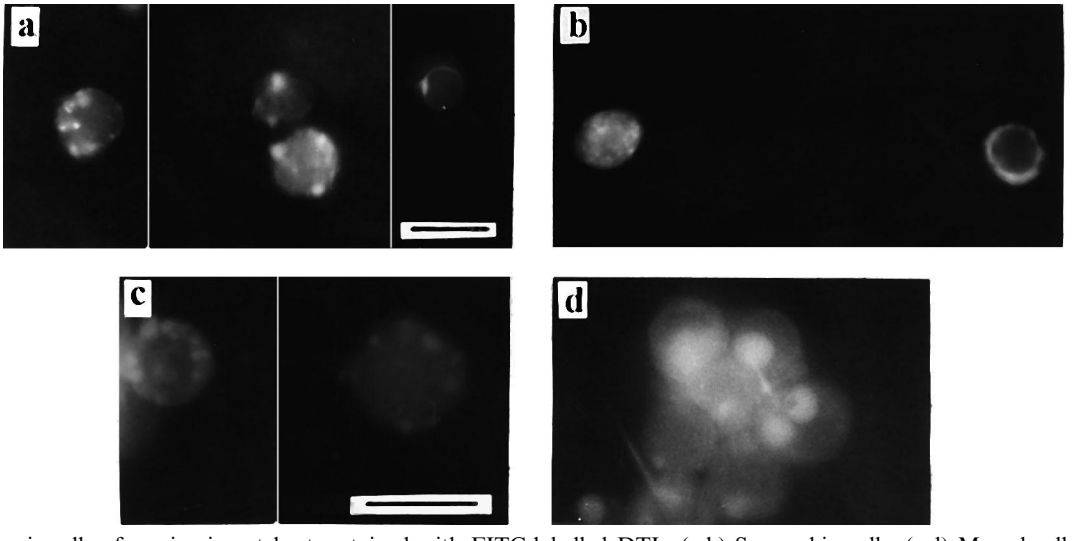

Fig. 5. Embryonic cells of marine invertebrates stained with FITC-labelled DTL. (a,b) Sea urchin cells. (c,d) Mussel cells. Cells at the blastula stage (a,c); at the gastrula stage (b); at the trochophore stage (d). Bar = 10 μm.

the collagen-binding site or RGD-sequence may be important for specific adhesive activity of DTL for embryonic cells. Earlier, RGD-sequence, which has been assumed to be a cell binding signal in cell-adhesive molecules [21], was found in echinoidin, a C-type lectin from the coelomic fluid of the sea urchin [22]. Echinoidin-induced cell adhesion was only inhibited by peptide GRGDS [23], suggesting that human cancer cells adhered to the echinoidin-coated substrate only through the RGD sequence. Similarly, RGD-dependent binding mechanism is involved in snail haemocyte responses [24]. In addition, some of the lectins are the hybrids of a collagen-like region and a lectin domain [25]. The fact of non-stage-specific expression of GlcNAc residues on the surface of molluscan and sea urchin cells suggests the possibility of the participation of non-lectin sites of DTL at the adhesion interaction with embryonic cells.

Glycoproteins may have a role not only as adhesion molecules but also as growth-promoting molecules with a role of bioregulators [26–28]. Mitogenic lectins from tissues of sponges were described earlier for human peripheral blood lymphocytes [29,30] and spleen lymphocytes from mice [31]. Moreover, several lectins and a mussel matrix protein contain EGF-like domains [32,33]. These data confirm the hypothesis of Engel [34] about localised signals for growth and differentiation, and accord with our results. In addition to adhesive activity, DTL has some characteristics of a growth factor stimulating the DNA synthesis in embryonic cells of sea urchins (and mussel cells, data not shown). The most intensive stimulation of the DNA synthesis was observed in the cell cultures obtained at the blastula stage when the adhesive effect of DTL was minimal. It is interesting that the other lectin, Con A, was shown to stimulate the DNA synthesis in only non-adherent cells of axial organ from the starfish *Asterias rubens* [35].

It should be remarked that at the blastula stage both DTL and Con A, in combination with insulin, significantly increased the RNA synthesis. Similarly, interleukin 1-like molecules from tunicates require costimulation with mitogenic lectins: namely, in the presence of a submitogenic dose of Con A there was a greater response in murine thymocytes [36]. The incubation of abalone haemocytes with insulin or epidermal growth factor resulted in the intensification of [^{3}H]leucine and [^{3}H]thymidine incorporation in primary culture, but only in the presence of Con A [37]. Unlike embryonic cells the haemocytes have a low proliferative potential; however, in the presence of lectins even haemocytes may reply to vertebrate growth factors.

DTL inducing 'capping-like' phenomena were observed at the cell surface of mollusc and sea urchin cells only at blastula stage. At this stage the lectin appeared as a growth factor. Capping phenomena were suggested to precede the mitogenic response of the cell [38]. It is known that development until the blastula stage depends on maternal information, while development at the gastrula stage is determined by the expression of embryonic genes [39,40]. After the mid-blastula stage embryonic cells become more sensitive to the signals of the environment.

Collectively, the lectin from the ascidian *Didemnum ternatanum* seems to have some characteristics of both an adhesive and a growth factor for cultivated molluscan and echinoderm cells. Recently, Kawamura and Fujiwara have established tunicate cell lines using Ca^{2+}-dependent, galactose-binding lectin from tunicate tissues with a remarkable anti-bacterial activity and cell growth activity [41]. The use of lectins may be a prospective method for the cultivation of marine invertebrate cells.

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